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MITOCHONDRIAL DYSFUNCTION AND TREATMENT STRATEGIES

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Mitochondrial Dysfunction and Treatment Strategies

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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It's the circle of life
It moves us all
Through despair and hope
Through faith and love
Till we find our place
In the path unwinding
In the circle of life

♥ *The Lion King*

வினைத்திட்பம் என்ப தொருவன் மனத்திட்பம்
மற்றைய எல்லாம் பிற

(Firmness in action is simply one's firmness of mind)

– Thiruvalluvar

Dedicated to **அப்பா** & **அம்மா** *(Dad and Mom)*

ABSTRACT

The mitochondria are essential for cellular energy production and are involved in many processes in the cells. The mitochondria contain their own DNA (mtDNA) that is vital for oxidative phosphorylation since it encodes enzymes of the respiratory chain. Mutations in the mtDNA and alterations in the mtDNA copy number are attributed to various human disorders including cancer. Mitochondrial DNA depletion syndromes (MDS) are a heterogeneous group of disorders characterized by severe depletion of the mtDNA. MDS predominantly manifests in high energy demanding tissues such as the skeletal muscle, brain and liver. Mutations in the genes that are responsible for providing precursors for the mtDNA synthesis such as thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) are known to cause MDS.

In an attempt to rescue the mtDNA depletion caused by thymidine kinase 2 (*Tk2*) deficiency in mice, the deoxyribonucleoside kinase from *Drosophila melanogaster* (*Dm*-dNK) was expressed in the *Tk2* deficient mice (*Dm-dNK*$^{+/-}$*Tk2*$^{-/-}$). The *Dm-dNK*$^{+/-}$ expression was able to rescue the *Tk2*$^{-/-}$ mice and prolong their life span from 3 weeks to up to 20 months. The *Dm-dNK* expression driven by the CMV promoter was observed in all tissues with highest expression in skeletal muscle and lower expression in heart, liver and adipose tissues. *Dm-dNK*$^{+/-}$*Tk2*$^{-/-}$ mice maintained normal mtDNA levels in the skeletal muscle and liver throughout the observation time of 20 months. The *Dm-dNK* expression resulted in highly elevated dNTP pools with dTTP pools being >100 times higher than in the wild type mice. However, the large increase in the dTTP pool did not cause mutations in the nuclear or the mitochondrial DNA. A significant reduction in total body fat (both subcutaneous and visceral fat) was observed only in the *Dm-dNK*$^{+/-}$*Tk2*$^{-/-}$ mice compared to wild type mice, which indicates an altered fat metabolism in these mice mediated through residual *Tk2* deficiency.

To elucidate effective treatment strategies for TK2 deficiency, a novel mouse model with liver specific expression of *Dm-dNK* driven by the albumin promoter was generated. Two founder mice with high *Dm*-dNK expression and activity in the liver was selected for further characterization. These mice will be used to study whether *Dm*-dNK expression in a single tissue would be able to rescue the sever phenotype caused by *Tk2* deficiency in mice.

The mitochondrial dicarboxylate carrier, SLC25A10, is involved in the transport of dicarboxylates such as malate and succinate across the mitochondrial inner membrane. To understand the role of the SLC25A10 carrier in regulating cancer cell growth, metabolism and transformation, a knockdown of SLC25A10 in a lung adenocarcinoma cell line (A549) was established and characterized. The growth properties of SLC25A10 knockdown cells changed to a less malignant phenotype, with increased dependency on glutamine and altered NADPH production. An increase in expression of glutamate dehydrogenase and decrease in expression of lactate dehydrogenase indicated a metabolic shift from glycolysis to oxidative phosphorylation in the SLC25A10 knockdown cells. The study demonstrates the importance of SLC25A10 in and regulation of redox homeostasis.

LIST OF SCIENTIFIC PAPERS

I. **Shuba Krishnan***, Xiaoshan Zhou*, João A. Parades, Raoul, V. Kuiper, Sophie Curbo, Anna Karlsson.
Transgene Expression of *Drosophila melanogaster* Nucleoside Kinase Reverses Thymidine Kinase 2 Deficiency.
J Biol Chem. **2013** Feb 15; 288(7):5072-5079.
*These authors contributed equally to this study

II. **Shuba Krishnan**, João A. Parades, Xiaoshan Zhou, Raoul, V. Kuiper, Kjell Hultenby, Sophie Curbo, Anna Karlsson.
Long Term Expression of *Drosophila melanogaster* Nucleoside Kinase in Thymidine Kinase 2-deficient Mice with no Lethal Effects Caused by Nucleotide Pool Imbalance.
J Biol Chem. **2014** Nov 21; 289(47):32835-32844.

III. **Shuba Krishnan**, Xiaoshan Zhou, Sophie Curbo, Anna Karlsson.
Construction of a mouse strain with liver specific expression *of Drosophila melanogaster* nucleoside kinase.
Manuscript

IV. Xiaoshan Zhou, João A. Paredes, **Shuba Krishnan**, Sophie Curbo, Anna Karlsson.
The mitochondrial carrier SLC25A10 regulates cancer cell growth.
Oncotarget. **2015** Apr 20; 6(11):9271-9283.

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LIST OF ABBREVIATIONS

adPEO	autosomal dominant PEO
bp	base pairs
CMV	cytomegalovirus
COX	cytochrome C oxidase
dCK	deoxycytidine kinase
dGK	deoxyguanosine kinase
Dm-dNK	*Drosophila melanogaster* nucleoside kinase
dNK	deoxyribonucleoside kinases
$FADH_2$	reduced form of flavin adenine dinucleotide
MDS	mitochondrial DNA depletion syndromes
MNGIE	mitochondrial neurogastrointestinal encephalomyopathy
MRC	mitochondrial respiratory chain complex
NADH	reduced form of nicotinamide adenine dinucleotide
NADPH	reduced form of nicotinamide adenine dinucleotide phosphate
NDPK	nucleotide diphosphate kinase
NMPK	nucleotide monophosphate kinase
OMIM	Online Mendelian Inheritance in Man
OxPhos	oxidative phosphorylation
P53R2	P53 inducible R2 subunit of ribonucleotide reductase
PEO	progressive external ophthalmoplegia
PNC	pyrimidine nucleotide carriers
Pol	polymerase
PRPP	phosphoribosyl pyrophosphate
RNR	ribonucleotide reductase
ROS	reactive oxygen species
SLC25	solute carrier family 25
TCA cycle	tricarboxylic acid cycle
TK1	thymidine kinase 1
TK2	thymidine kinase 2

Nucleosides, nucleotides and nucleic acids

A, G, T, C	adenine, guanine, thymine, cytosine
dN	deoxyribonucleoside
Ado, dAdo	adenosine, deoxyadenosine
Cyt, dCyt	cytidine, deoxycytidine
dThd	deoxythymidine
Guo, dGuo	guanosine, deoxyguanosine
Urd, dUrd	uridine, deoxyuridine
dA, dAMP, dADP, dATP	deoxyadenosine, mono-, di- and tri- phosphate
dC, dCMP, dCDP, dCTP	deoxycytidine, mono-, di- and tri- phosphate
dG, dGMP, dGDP, dGTP	deoxyguanosine, mono-, di- and tri- phosphate
dT, dTMP, dTDP, dTTP	deoxythymidine, mono-, di- and tri- phosphate
dU, dUMP, dUDP, dUTP	deoxyuridine, mono-, di- and tri- phosphate

ATP	adenosine triphosphate
NMP, NDP, NTP	any ribonucleoside mono-, di- and tri- phosphate
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
nDNA	nuclear DNA
mtDNA	mitochondrial DNA

Genes

C10ORF2	twinkle helicase
DCK	deoxycytidine kinase
DGUOK	deoxyguanosine kinase
MPV17	mitochondrial inner membrane protein 17
mt-Cytb	mitochondrial cytochrome b
NCR	non-coding region
POLG	polymerase gamma
RRM1, RRM2	ribonucleotide reductase subunit M1 and M2
RRM2B	p53 inducible ribonucleotide reductase subunit 2
SUCLG1, SUCLA2	succinate coenzyme A ligase α- and β- subunits
TK1	thymidine kinase 1
TK2	thymidine kinase 2
TYMP	thymidine phosphorylase
UPP	uridine phosphorylase

1 INTRODUCTION

Abnormalities in the genome, with subsequent alterations of gene functions, are common causes of diseases. Genetic diseases may be hereditary or caused by new mutations or alterations in the DNA. Sometimes a single gene is mutated but more often genetic disorders are complex with involvement of several genes and effects of lifestyle and environmental factors. Mitochondrial dysfunction constitutes a large group of genetic inherited metabolic disorders which are due to deficient energy production by the mitochondrial respiratory chain complex. Genetic defects in both the nuclear and the mitochondrial DNA or defects in the inter-genomic signaling can result in mitochondrial dysfunction. Mitochondrial dysfunction contributes to a wide variety of diseases including neonatal fatality, adult onset neurodegeneration and cancer (1). Mitochondrial DNA depletion syndrome is characterized by reduced levels of mitochondrial DNA that largely affects infants and children and leads to early death (2). Deficiencies in enzymes that participate in the DNA precursor synthesis are among the genetic disorders that cause mitochondrial DNA depletion syndromes. Furthermore mitochondria are known to play an important role in the regulation of cell proliferation and cell death and are involved in the altered metabolism of cancer cells. To elucidate metabolic pathways that are important in cancer cell proliferation is a way to identify novel targets for cancer therapy.

2 THE DNA MOLECULE

Deoxyribonucleic acids (DNA) are the molecules that carry the genetic information of life. DNA are long polymers made of repeating units called deoxyribonucleotides that are arranged in specific triplets that make up the genetic code. Deoxyribonucleotides are composed of a nitrogenous purine or pyrimidine nucleobase, bound to a 5-carbon deoxyribose sugar and one, two or three phosphate groups. The nucleobases are classified into two groups; the purines adenine (A) and guanine (G), and the pyrimidines cytosine (C), and thymine (T). The DNA molecule is a double stranded helical molecule, containing millions of bases linked to each other by phosphodiester bonds and between each other by hydrogen bonds (3). Bases A and T and bases C and G form double or triple hydrogen bonds with each other respectively (figure 1).

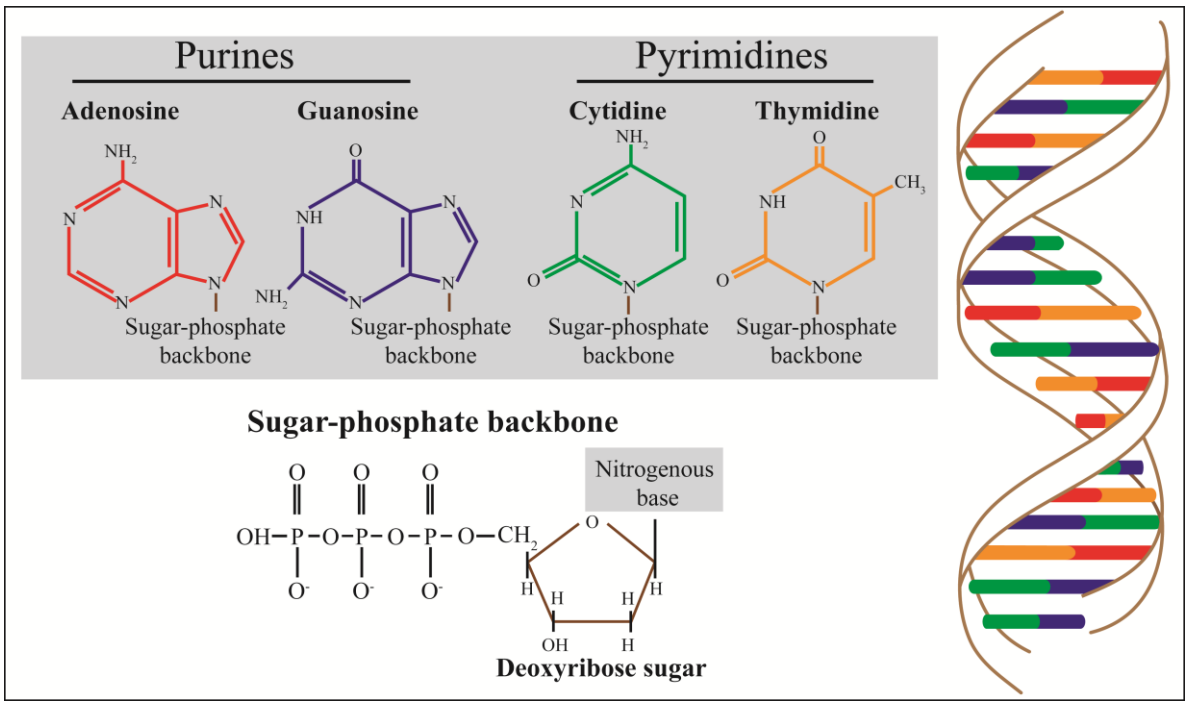

Figure 1: Deoxyribonucleotide structure

2.1 THE HUMAN GENOME

The human genome includes DNA present in two organelles in the cell; the nuclear DNA (nDNA) and the mitochondrial DNA (mtDNA). The majority of the genetic information in eukaryotic cells is encoded in the nDNA, while the mtDNA constitute about 2-3% of the total genome and encodes proteins essential for the synthesis of ATP.

2.1.1 The nuclear DNA

The nDNA consists of linear, double stranded molecules that form double helical structures and are tightly packed within the nucleus of a cell. Humans have a diploid genome containing approximately 3 million base pairs (bp) packed in 23 pairs of chromosomes. Chromosomes can be classified as autosomes and allosomes (sex chromosomes). Chromosomal pairs 1-22 in the human genome are autosomal while allosomes are the X and the Y chromosomes (XX for female and XY for male).

DNA replication is the process where novel DNA molecules are synthesized from deoxyribonucleoside triphosphates (dNTPs), using one strand as template. The two strands of the DNA double helix unwind with the help of helicases and form a replication fork (two single stranded templates) that each serve as templates for the synthesis of daughter strands. Several DNA polymerases exist that assist the DNA synthesis by addition of correct dNTPs to synthesize a complementary strand.

The nDNA synthesis is a tightly regulated process and occurs in specific phases of the cell cycle. In the G_1 phase the replication process is initiated and all the proteins and factors required for synthesis are assembled. The majority of nDNA synthesis in eukaryotic cells occurs during the S phase of the cell cycle. Following the S phase, the G_2 phase checks for damage or errors during DNA synthesis and prepares the cell for mitosis to form 2 daughter

cells. The daughter cells enter the G_1 phase again and the process repeats itself. Cells can also enter a non-dividing state, which is either not reversible (senescence or apoptosis), or a non-proliferative phase (G_0 phase, resting phase or quiescence). There are several check points in the cell cycle that ensures that DNA synthesis occurs without errors (4).

2.1.2 The mitochondrial DNA

Mitochondria contain their own DNA, the mtDNA, which contains genes encoding respiratory chain complexes. Compared to the linear nDNA, the mtDNA are circular double stranded molecules (16.5 kbp), that contain only 37 genes. 100-10,000 copies of mtDNA are present in each cell depending on the energy requirement of the specific cell. The mtDNA replication is independent of the cell cycle and the nDNA replication. Unlike nDNA that divides during the S phase of the cell cycle, mtDNA replication occurs asynchronously throughout the cell cycle and also occurs in post-mitotic resting cells such as brain and muscle cells. The mtDNA replication requires a constant supply of dNTPs and is regulated by several nuclear encoded proteins such as DNA polymerase γ (POLG), twinkle helicase and mitochondrial single-stranded DNA binding proteins (5-8).

2.2 THE SYNTHESIS OF DEOXYRIBONUCLEOTIDES

Depending on the cell cycle phase, cells can be classified as dividing cells or resting cells. In addition to the different enzymes and co factors, an adequate supply of dNTPs (dATP, dCTP, dTTP and dGTP), are required for DNA synthesis and repair in both dividing and resting cells. There are two tightly regulated pathways for nucleotide biosynthesis; the *de novo* pathway and the salvage pathway (figure 2).

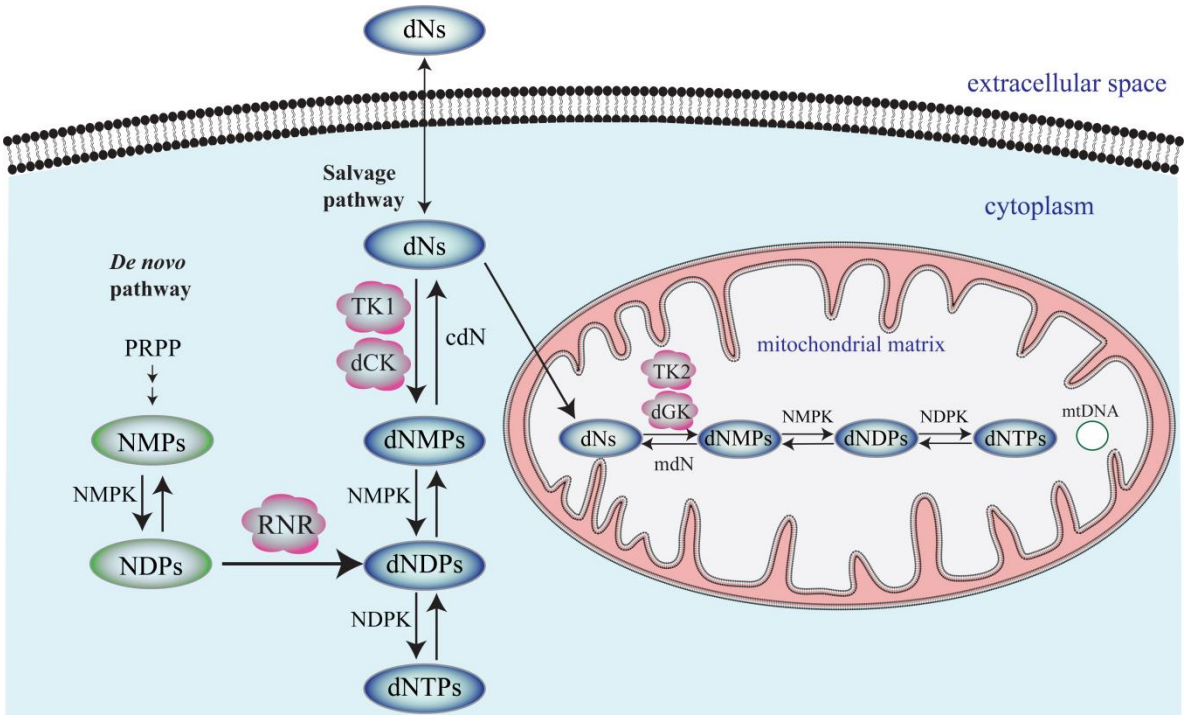

Figure 2: Deoxyribonucleotide synthesis in the cytosol and mitochondria

The *de novo* pathway assembles ribonucleotides from sugars and amino acids. Ribose-5-phosphate, a product of glucose breakdown via the pentose phosphate pathway, reacts with ATP to generate the activated form, phosphoribosyl pyrophosphate (PRPP). PRPP, along with amino acids and carbon dioxide forms inositol monophosphate, which is subsequently converted to adenosine and guanosine monophosphates. Pyrimidines are assembled using bicarbonates, ATP, glycine and coenzyme Q, and are finally attached to PRPP to form uridine monophosphate. Nucleoside monophosphates are reversibly phosphorylated to nucleoside diphosphates (NDPs) catalyzed by nucleotide monophosphate kinases (NMPK). In humans, there are different NMPKs for the different nucleosides; thymidylate kinase, uridylate-cytidylate kinase, several isoforms of adenylate and guanylate kinases (9,10).

The enzyme ribonucleotide reductase (RNR) catalyzes the conversion of NDPs to deoxyribonucleoside diphosphates (dNDPs) by reduction of nucleotides in the 2-hydroxyl group of the sugar moiety (11). Since DNA requires thymidine deoxyribonucleotides, dUMP is converted to dTMP through reductive methylation catalyzed by the enzyme thymidylate synthase. The dNDPs are subsequently phosphorylated into respective dNTPs catalyzed by the enzyme nucleoside diphosphate kinase (NDPK) (12). In humans there are several NDPK isozymes, differentially expressed in tissues, possessing several different yet specific functions in the cell (13,14).

The salvage pathway employs enzymes known as deoxyribonucleoside kinase (dNK) to catalyze the phosphorylation of deoxyribonucleosides that are either recycled from degraded DNA or obtained from nutrients. Mammals have four dNKs with specific but overlapping substrate specificities and all encoded by the nDNA (15,16) (Table 1). Two of the enzymes are cytosolic; thymidine kinase 1 (TK1), and deoxycytidine kinase (dCK), and two of the enzymes are located in the mitochondria; thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK). The phosphorylation of deoxyribonucleosides to dNMPs by dNKs is the first and rate-limiting step in the salvage pathway. The dNMPs are subsequently phosphorylated to di- and tri- phosphates by NMPK and NDPK respectively.

Table 1: The four mammalian dNKs, their substrates and expression

Enzyme	**Human gene**	**Subcellular localization**	**Substrates**	**Expression pattern**
TK1	*TK1*	cytosol	dThd, dUrd	S phase
TK2	*TK2*	mitochondria	dThd, dUrd, dCyt	Constituent
dCK	*DCK*	cytosol	dCyt, dGuo, dAdo	Constituent
dGK	*DGUOK*	mitochondria	dAdo, dGuo	Constituent

2.2.1 The synthesis of dNTPs in dividing cells

The major source of dNTPs for DNA synthesis in dividing cells are from the *de novo* pathway, where the RNR catalyzes the rate limiting step in DNA synthesis. RNRs are tetrameric proteins containing two non-identical subunits: a large regulatory subunit R1 and a

small catalytic subunit R2 (17-19). In dividing cells, enzyme levels and activity of the R1 subunit is constant throughout the cell cycle, while the R2 subunit is cell cycle (S phase) regulated (18,20,21). Another source of dNTPs in dividing cells is via the salvage pathway mediated by the dNKs. TK1 activity is cell cycle regulated, and is highly S-phase specific, while dCK, dGK and TK2 are constitutively expressed throughout the cell cycle (15,22). Supply of dNTPs for mtDNA synthesis in dividing cells are likely both from the *de novo* and salvage pathways.

2.2.2 The synthesis of dNTPs in resting cells

Resting cells do not undergo cell division and therefore do not have nDNA synthesis. However, mtDNA is constantly replicating and requires dNTP supply also in resting cells. The two constituently expressed dNKs that are located in the mitochondria, TK2 and dGK, supply the required dNTPs for mtDNA synthesis in resting cells (23-27). Furthermore, the nDNA is constantly subjected to DNA damage and also requires an adequate supply of dNTPs for DNA repair. Expression of the R2 subunit of RNR and TK1 are undetectable in resting cells (28-30). The cytosolic/nuclear dCK phosphorylates dAdo, dGuo and dCyt deoxynucleosides (31).

The *P53* is a tumor suppressor gene and has been shown to be inactivated in a wide range of cancers (32,33). A p53 inducible ribonucleotide reductase subunit (p53R2) is similar to the R2 subunit of RNR (34). The p53R2 is expressed throughout the cell cycle and shown to supply dNTPs to both mtDNA and nDNA in resting cells in response to various radiation and genotoxic stress causing DNA damage (35-38).

2.3 GENOMIC STABILITY AND dNTP BALANCE

DNA synthesis requires an adequate and balanced supply of dNTPs in order to function normally (19,39,40). Abnormal dNTP pools are known to cause mutagenic phenotypes, and correctly regulated dNTP pools are therefore a critical factor in maintaining genomic stability (41,42). The mtDNA corresponds to a small percentage of the total DNA and only small amounts of precursors are needed in comparison to the much larger nuclear DNA. Due to these differences mtDNA maintenance needs small but continuously present dNTP levels whereas nuclear DNA replication demands higher levels but restricted to the S phase of the cell cycle (43).

To keep the dNTP pools balanced their synthesis is regulated either by allosteric regulation of RNR or feedback inhibition of dNKs (19,44), or by substrate cycles involving catabolic enzymes that degrade deoxynucleotides (19). Several enzymes such as nucleotidases, purine nucleoside phosphorylases, adenosine deaminase, thymidine phosphorylase, sterile α motif HD-domain containing protein 1 and uridine phosphorylase are catabolic enzymes that breakdown deoxyribonucleotides and deoxyribonucleosides to smaller bases and sugars that can either be excreted through the urine or recycled in the cell for different cellular processes. Mutations or deletions in any of the enzymes involved in the

regulation of the dNTP pool balance affects the fidelity of DNA synthesis and contribute to a variety of human disorders (40,45).

The mitochondrial dNTP pool is separated from the cytosolic pool by the mitochondrial inner membrane, which is impermeable to charged molecules (46,47). However, several studies show a communication between the cytosolic and the mitochondrial dNTP pools with transporters present in the inner mitochondrial membrane (48-50). The equilibrative nucleoside transporters are located in both the plasma membrane as well as the mitochondrial inner membrane where they facilitate deoxyribonucleoside transport into the mitochondria (51,52). Mitochondrial pyrimidine nucleotide carriers (PNC1, PNC2) transport deoxyribonucleotide di- and tri- phosphates across the mitochondrial matrix (53,54). In dividing cells, cytosolic dNTP pools are high and the mitochondria can access the cytosolic dNTP pools through the PNC carriers. However in resting cells, cytosolic dNTP pools drop and mitochondria depend on the mitochondrial TK2 and dGK. The availability of balanced dNTP pools within mitochondria is important for mitochondrial genome integrity and stability, and an imbalance interferes with normal mtDNA replication and repair processes leading to mtDNA depletion (55). Imbalances in dNTP pools may cause mutations in both mitochondrial and nuclear DNA and are associated with several human disorders including cancer (56).

3 MODELS TO STUDY GENETIC DISEASES

3.1 THE *Drosophila melanogaster* MODEL ORGANISM

Drosophila melanogaster is one of the most commonly and intensively studied organisms in biology, especially in genetics. It has a short life cycle, and serves as a model to study several cellular and developmental processes that are common to higher eukaryotes. It has a small genome compared to humans with only 4 pairs of chromosomes (X/Y, II, III and IV) Most of the genetic information is present in chromosomes X, II and III (57). Approximately 60% of human disease genes are conserved in *Drosophila melanogaster* (58).

The mtDNA of *Drosophila melanogaster* is a 19.5 kb molecule and is similar to mammalian mtDNA, although with different gene arrangements. The non-coding region (NCR) in *Drosophila* mtDNA is an A+T rich region and it is of different size in different *Drosophila* subgroups (59).

In *Drosophila melanogaster,* four classes of DNA polymerases have been identified and characterized; pol-α, γ, δ and ε. Pol-α, δ, and ε are involved in nDNA synthesis and function throughout nDNA replication and repair while pol-γ is involved in mtDNA synthesis (60-63).

Drosophila melanogaster has a single multisubstrate deoxynucleoside kinase (*Dm*-dNK) that has the ability to catalyze the conversion of all four deoxynucleosides to their respective monophosphates (64,65). Cloning and characterization of *Dm*-dNK showed that this 29 kDa enzyme has high sequence similarity to mammalian TK2 and is closely related also to dGK and dCK (66,67). Preferred substrates for *Dm*-dNK are dThd, dUrd and dCyt,

but purine nucleosides are also efficiently catalyzed. *Dm*-dNK has 4-20,000 fold higher catalytic activity than mammalian dNKs, depending on the substrate (68).

3.2 THE MOUSE (*Mus musculus*) MODEL ORGANISM

The past century has seen rapid development in use of laboratory mice as model organisms to study different areas of human diseases. Although yeast, worms and flies are exceptional models to study developmental biology and genetics, mice have served as better tools to study cardiovascular, nervous, immune, and other complex mammalian diseases. From immunosuppressed mouse models to humanized mouse models, there are thousands of different types of unique, exclusive and rare inbred and genetically modified strains that are used in almost every field of biological research.

Mice have 20 pairs of chromosomes (19 pairs autosomal, 1 X/Y pair) and the mouse genome was sequenced in 2002. The mouse genome is 14% smaller than the human genome; however over 80% of the mouse genes have a corresponding human counterpart (69). Both mice and humans have similar amounts of protein coding genes. Mouse mtDNA is highly homologous to human mtDNA, with respect to overall gene organization and sequence (70).

Common models to study human disease mechanisms are to use knockout, knockin or transgenic mice. Generation of knockout and knockin mouse models involve genetic manipulation of a specific locus in embryonic stem cells via homologous recombination. Knockout mice are generated by targeting a specific gene locus and rendering it non-functional by deleting or disrupting the gene. Knockout mice are commonly used to study human gene deficiencies. Knockin mice are generated by targeted insertion of a gene at a specific locus under the regulatory elements of another gene. Alternatively, transgenic mice are generated via random integration of a transgene construct into the mouse genome by injecting the transgene into the pronuclei. Both transgenic and knockin mouse models are used to study effects of gene overexpression. The expression of the transgene must be driven by its own strong promoter. Unlike homologous recombination, which is targeted integration into the genome, pronuclear injection causes random integration and variability in transgene copy number. (71,72).

4 MITOCHONDRIA

Mitochondria, known as the ‘power house of the cells’, are complex, dynamic, semiautonomous, double-membraned organelles within almost all eukaryotic cells that provide energy through the process of oxidative phosphorylation (OxPhos). Evidence supports that billions of years ago, mitochondria were aerobic free living bacteria that were engulfed by a host cell, which has evolved to become the present day eukaryotic cell (73). Every cell has 10-1000s of mitochondria depending on the energy requirement of the cell. There are two pathways for ATP synthesis; glycolysis and OxPhos. Glycolysis involves breakdown of 6-carbon glucose to 3-carbon pyruvate. The pyruvate is converted to acetyl coenzyme A (acetyl CoA) which enters the mitochondria to participate in the tricarboxylic

acid cycle (TCA cycle) that produces the energy precursors for OxPhos. Electron carriers such as the reduced forms of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$), produced during glycolysis and the TCA cycle, are involved in transporting electrons from one component to another to generate ATP in the mitochondria. In addition, mitochondria have important roles in apoptosis, ageing, calcium signaling, iron-sulphur cluster assembly, iron metabolism and innate and adaptive immunity (74-82).

4.1 MITOCHONDRIAL DNA DEPLETION SYNDROMES

Human mtDNA is a circular double stranded DNA containing 16,569 bp encoding 37 genes; 13 protein coding genes, 22 transfer RNA coding genes and 2 ribosomal RNA coding genes (70,83,84). Various studies have contributed to the knowledge that mammalian mitochondria contain around 1,500 proteins that are expressed in a tissue specific manner (85,86). Since the mtDNA encodes only 13 of those proteins the mitochondria depend on nuclear DNA for all other proteins. The 13 protein encoding genes in the mitochondria encode enzymes involved in the OxPhos therefore an intact mtDNA is crucial for ATP production. Unlike nDNA, human mtDNA do not contain introns, and have almost the entire NCR concentrated in 1 region. The NCR contains elements for transcription and two origins of replication (87,88).

Mitochondrial DNA depletion syndromes (MDS) are clinically heterogeneous autosomal recessive disorders characterized by severe reduction in mtDNA levels (2,89,90). Mutations in nuclear encoded genes involved in the nuclear-mitochondrial crosstalk are also associated with mtDNA depletion syndromes (2,91). The mtDNA depletion may affect either a specific tissue or a combination of organs and tissues, including muscle, liver, brain and kidney (92). MDS manifestation can therefore be classified into four different forms: 1) myopathic, 2) hepatocerebral 3) encephalomyopathic and 4) neurogastrointestinal (2). Functional defects in any of the genes involved in mtDNA synthesis or maintenance results in mtDNA depletion. The most common cause of MDS are mutations in nuclear encoded *TK2*, *DGUOK*, P53R2 *(RRM2B)*, thymidine phosphorylase (*TYMP*), succinyl coenzyme A ligase alpha (*SUCLG1*) and beta (*SUCLA2*) subunits, enzymes that regulate dNTPs pools in the mitochondria (2). Additionally, defects in other proteins responsible for mtDNA replication and maintenance such as polymerase γ (*POLG*), the twinkle helicase (*C10ORF2*), and mitochondrial inner membrane protein (*MPV17*) can also cause mtDNA depletion (2,91,93). MDS can be difficult to diagnose since many tissues can be simultaneously affected and the prevalence of MDS is unknown.

4.1.1 Thymidine kinase 2

Mitochondrial TK2 is encoded by the nuclear *TK2* gene (chromosome 16) and phosphorylates the pyrimidine deoxyribonucleosides, dThd, dUrd and dCyt to their respective monophosphates (25). TK2 deficiency is associated with a myopathic form of MDS (OMIM: #609560) (94). The first reports of children affected by TK2 deficiency had severe infantile myopathy and most often presented with gradual onset of hypotonia, fatigue, feeding

difficulties, proximal muscle weakness and loss of previously acquired motor skills (94-98). However, recent reports have identified milder forms of TK2 deficiency with adult onset and longer survival (99,100). TK2 deficiency can cause multi-organ mtDNA depletion with manifestation in muscle, brain and liver (101,102). Approximately 45-50 individuals have been reported with TK2 deficiency (103,104). Clinical diagnosis of TK2 deficiency includes multiple ragged red fibers, cytochrome C oxidase (COX) negative fibers, elevated serum creatine kinase levels and depletion of mtDNA content in the muscle biopsy sample (75-85%).

4.1.2 Deoxyguanosine kinase

Mitochondrial dGK is encoded by the nuclear *DGUOK* gene (chromosome 2) and phosphorylates the purine deoxyribonucleosides, dGuo and dAdo to their respective monophosphates (24). dGK deficiency is associated with the hepatocerebral form of MDS (OMIM: #251880) (105). Most of the individuals with *DGUOK* deficiency have severe multi-organ illness, with progressive liver damage, hypoglycemia, lactic acidosis and neurological damage (105-110). Approximately 100 individuals have been clinically diagnosed with *DGUOK* deficiency (110,111). The cytoplasmic enzyme dCK has overlapping substrate specificity with dGK phosphorylating dAdo, dGuo and dCyt in the cytoplasm (15,112). The tissue specificity of dGK deficiency is likely due to low expression of dCK in brain and liver tissues, which have a high demand of functioning mitochondria. Therefore these tissues depend on dGK for supply of precursors for mtDNA synthesis (105).

4.1.3 Thymidine phosphorylase

Thymidine phosphorylase is a cytosolic enzyme encoded by the nuclear *TYMP* gene (chromosome 22), which catalyzes the breakdown of dThd and dUrd to thymine or uracil respectively. The protein was initially identified as platelet-derived endothelial cell growth factor (113) and was thought to be specific to endothelial cells. Later on the *TYMP* gene expression was detected in other tissues with highest expression found in lung, brain, spleen and the digestive system, and relatively lower expression in kidney, muscle and fat (114). Additionally, the protein showed to have angiogenic activity in mouse tumors with 4-5 times higher expression in tumor cells than normal cells (113,114), and to catalyze the reversible dephosphorylation of thymidine to thymine (115).

Mutations in *TYMP* cause MDS manifesting as mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) (OMIM: #603041) (116). MNGIE is a progressive multisystem disorder characterized by severe ptosis, progressive external ophthalmoplegia (PEO), gastrointestinal dysmotility, cachexia, diffuse leukoencephalopathy, peripheral neuropathy, ragged-red fibers or increased succinate dehydrogenase activity in muscle biopsy, and mitochondrial dysfunction caused by mtDNA depletion, deletions and point mutations (116-118). Severe impairment of the TYMP enzyme causes increased accumulation of plasma thymidine which disturbs the mitochondrial dNTP pool balance leading to mtDNA abnormalities (119).

4.1.4 P53 inducible subunit of ribonucleotide reductase

The P53 inducible ribonucleotide reductase small subunit (P53R2) is encoded by the nuclear *RRM2B* gene (chromosome 8) that forms a functional RNR with the R1 subunit and catalyzes the conversion of NDPs to dNDPs (34,120). Human cancers are frequently characterized by inactivation of the P53 gene, that in a normal cell acts as a tumor suppressor by regulating the cell cycle or inducing apoptosis (33). The P53R2 subunit has high sequence similarity with the R2 subunit of the RNR, and has a crucial role in regulating dNTP synthesis during DNA damage (34,37,121). *RRM2B* was shown to be constitutively expressed in low amounts in proliferating and resting cells (36).

RRM2B is ubiquitously expressed in human tissues with high expression in skeletal muscle. *RRM2B* gene mutations primarily cause myopathy (OMIM: #612071), characterized by severe mtDNA depletion (1-10% of controls) ragged red fibers and COX negative staining, but also affects kidney and brain (122-125).

4.1.5 Succinyl coenzyme ligase subunits α and β

Succinyl coenzyme A synthetase, also known as succinate coenzyme A ligase (SUCL), is a mitochondrial TCA cycle enzyme that catalyzes the reversible reaction between succinyl coenzyme A and succinate. Succinyl CoA is important for activation of ketone bodies and heme synthesis. There are two isoforms of SUCL; an ATP-specific isoform (SUCLA) and a GTP-specific isoform (SUCLG) catalyzing ATP and GTP dependent reactions respectively (126).

SUCL proteins are composed of 2 subunit types; an α-subunit encoded by *SUCLG1* and a β-subunit encoded by either *SUCLA2* or *SUCLG2* that determines the nucleotide specificity of the enzyme (126-129). The β-subunits are expressed in most human tissues with *SUCLA2* being predominantly expressed in brain and muscle and *SUCLG2* in liver and kidney. *SUCLG1* is ubiquitously expressed with highest expressions in brain, heart, kidney and liver (126,129,130). Mutations in *SUCLA2* and *SUCLG1* are associated with hepatoencephalomyopathic forms of MDS (with methylmalonic aciduria) (OMIM: #245400) (131). Deficiency of SUCLA2 results in Leigh's or a Leigh-like syndrome with onset of severe hypotonia before the age of 6 months. Affected children develop sensorineural hearing impairment, psychomotor delay, and severe muscular atrophy (132). Most patients die in childhood, and metabolic analysis consistently shows elevated levels of plasma and urine methylmalonic acid (128,133-136). Mutations in *SUCLG1* causes fatal infantile lactic acidosis and affects the liver (40% of the patients), manifesting as hepatomegaly, steatosis, and liver failure (129,137-139). 15% of patients with *SUCLG1* mutations also present with hypertrophic cardiomyopathy (129). Approximately 70 patients with *SUCLA2* and *SUCLG1* mutations have been clinically diagnosed so far (129).

4.1.6 Polymerase gamma

DNA polymerase gamma (pol γ) plays an important role in mitochondrial DNA replication and repair (140). Human pol γ is highly expressed in skeletal muscle and heart

tissues (141). The pol γ holoenzyme contains two subunits; the catalytic subunit encoded by the nuclear *POLG* gene that has DNA polymerase, 3′-5′ exonuclease and 5′-deoxyribose phosphate lyase activities, and an accessory subunit encoded by the nuclear *POLG2* gene that participates in DNA binding and DNA synthesis (142-144).

Over 200 mutations in *POLG* have been identified that are associated with Alpers-Huttenlocher syndrome, childhood myocerebrohepatopathy spectrum disorders, myoclonic epilepsy myopathy sensory ataxia, ataxia neuropathy spectrum of syndromes and PEO (OMIM: #203700, #613662) (reviewed in (145,146)). Multiple tissues such as liver, skeletal muscle and brain are affected and have been shown to have mtDNA depletion. The onset of the clinical phenotype can vary from early infantile to late adult onset as in the case of PEO and ataxia neuropathy syndromes (147).

4.1.7 Twinkle helicase

Twinkle is a mitochondrial protein encoded by the nuclear *C10ORF2* gene (chromosome 10 open reading frame 2). Twinkle was identified as a T7 phage helicase like protein, and the name derives from the localizing pattern resembling twinkling stars (148). Mutations in *C10ORF2* are associated with a hepatocerebral form of MDS (OMIM: #271245) and cause autosomal dominant PEO (adPEO) and infantile-onset spinocerebellar ataxia (148,149). Common clinical features of adPEO include hearing loss, proximal muscle weakness, ptosis, ophthalmoplegia and sensory axonal neuropathy (150). Some patients with late onset PEO also developed dementia in their late seventies (151). The spinocerebellar ataxia phenotype was characterized by severe mtDNA depletion in brain and liver, progressive cerebellar atrophy, sensory axonal neuropathy, severe neonatal hypotonia increased serum lactate levels, seizures and peripheral neuropathy (152,153).

4.1.8 MPV17

MPV17 is a human gene that encodes a mitochondrial inner membrane protein, believed to play a role in the metabolism of reactive oxygen species (ROS) (154). The MPV17 protein is expressed in human liver, heart, kidney, skeletal muscle, lung, brain, pancreas and placenta (155). *MPV17* mutations were initially identified as Navajo neurohepatopathy, where affected children presented with sensorimotor neuropathy, spinal cord atrophy, corneal ulceration, acral mutilation, progressive central nervous system white matter lesions and liver disease (156,157). Mutations in *MPV17* is associated with hepatocerebral forms of MDS (OMIM: #256810), characterized by progressive liver failure, often affecting children within their first year of life (154,155,158,159).

4.1.9 Other genes causing MDS

The clinical spectrum of MDS is expanding and new genes and novel mutations in previously described genes are constantly emerging. Several new genes have been identified whose mutations cause severe mtDNA depletions and mtDNA deletions (OMIM: #212350; #615084; #616896; #615471). Mutations in acylglycerol kinase (a mitochondrial membrane

protein involved in lipid and glycerolipid metabolism), mitochondrial genome maintenance exonuclease 1 gene, mitochondrial dynamin like GTPase, mitochondrial F-box, leucine-rich repeat 4 protein, and mitochondrial transcription factor A have recently been associated with cardiomyopathic, encephalomyopathic and hepatocerebral forms of MDS (160-166).

4.2 ANIMAL MODELS FOR MDS

Animal models are important tools to develop treatments for rare diseases particularly when only small populations of patients are available to evaluate the disease. The use of animal models helps unveil the natural history of the disease, its etiology and characteristics. Modelling mitochondrial dysfunction and mtDNA depletion syndrome is particularly difficult due to the unique mitochondrial genetics. Different animal models, mainly mouse and rats, have been generated to study MDS with the aim to gain mechanistic insights and to develop therapeutic strategies.

TK2 deficiency: Two different mouse models have been generated for TK2 deficiency; a knockin mouse model with the amino acid substitution H126N, corresponding to the human pathogenic mutation H121N (167), and a *Tk2* knockout mouse model with a deletion of exon 4 and part of exon 5, which encodes for the substrate binding domain of the enzyme active site (168). Both the *Tk2* deficient mouse models, despite the genetic differences, had normal growth at birth, and progressive growth decline from postnatal day 7 to 10. Both models displayed encephalomyopathy and neurological involvement caused by severe mtDNA depletion in the brain, and died prematurely within 2-4 weeks of age (167-169). The *Tk2* knockout mice had severe hypothermia and loss of hypodermal fat. A mtDNA depletion was observed in adipose tissues, causing alterations in brown and white adipose tissue development (170). Tissue specificity and onset of TK2 deficiency has been attributed to transcriptional compensation of TK1 (171). Gradual depletion of mtDNA in mouse liver was also observed (up to 80% reduction by postnatal day 14), that was accompanied by increased mitochondrial volume, altered mitochondrial structure in the liver, reduced mitochondrial β-oxidation and accumulation of lipid vesicles in the liver cells (172).

dGK deficiency: A rat model for dGK deficiency was reported recently (173). The *Dguok* deficient rats were generated using zinc finger nuclease technology that generated 3 knockout rat lines with varied base pair deletions causing approximately 90% reduction in hepatic mtDNA in these rats. The mtDNA depletion was also observed in spleen although to a lesser extent (60% of control). Muscle had no reduction in mtDNA content but had 20-30% COX negative fibers, and reduced complex I and III protein expressions. Electron paramagnetic (spin) resonance spectroscopy technique was applied to characterize respiratory chain abnormalities in the dGK deficient rats (173). The rats did not show a MDS phenotype suggesting a remaining dGK activity in this rat model.

TYMP deficiency: A knockout mouse model with targeted deletion of both thymidine and uridine phosphorylase (*Tymp*$^{-/-}$*Upp*$^{-/-}$) showed hyperintense brain lesions and axonal swelling

indicative of mitochondrial leukoencephalopathy (174). Increased levels of dThd, Urd and dUrd were measured in plasma and several tissues including skeletal muscle, brain, kidney and heart of *Tymp*$^{-/-}$*Upp*$^{-/-}$ mice (175). The severe progressive mtDNA depletion in these mice is due to unbalanced dNTP pools caused by *Tymp Upp* deficiency, and reflects the MNGIE patient phenotype (175,176).

P53R2 deficiency: *Rrm2b* knockout mice were generated by deleting exon 3 and 4 of the *Rrm2b* gene. *Rrm2b*-deficient mice appeared normal at birth and displayed progressive growth retardation from 6 weeks of age, followed by premature death due to renal failure (approximately 12 weeks) (177). The renal failure in *Rrm2b*-deficient mice is caused by alterations of dNTP pools causing oxidative stress and increased spontaneous mutations (177). Other organs including heart, skeletal muscle, liver and nerve fibers also underwent atrophic changes.

POLG deficiency: Several mouse models have been generated with modified *Polg*. *PolgA* knockout mice showed early developmental arrest between embryonic day E7.5-8.5 associated with severe mtDNA depletion (178). Transgenic mice with specific cardiac tissue targeted mutants of human Pol γ, (Y955C point mutation), caused chronic PEO in the heart, with cardiomyopathy, mitochondrial oxidative stress and structural damage, pathological cardiomegaly, and premature death (179). *PolG2* mutations are known to cause adPEO. A *Polg2* knockout mouse model, generated to better understand the functions of POLG2, resulted in embryonic lethality (E8.0-8.5) with mtDNA depletion and mitochondrial ultrastructural defects (180). Several other mouse models for *Polg* mutations have been developed and extensively studied. These "Mutator mice" have impaired 3'-5' exonuclease activity thereby causing accumulation of mtDNA deletions and point mutations leading to OxPhos deficiency, and a premature ageing phenotype (181-186).

SUCLA2 and SUCLG1 deficiency: Transgenic mice were generated using a mutant allele of *Sucla2* isolated by FACS-based retroviral-mediated gene trap mutagenesis screen that identified abnormal mitochondrial phenotypes in mouse ES cells (187). Homozygotes with mutant *Sucla2* transgene were embryonically lethal (E18.5) with varying mtDNA depletion in embryonic brain, heart and muscle tissues (20-60% of control). Currently there is no animal model reported with *Suclg1* deficiency.

Twinkle helicase deficiency: A conditional gene knockout mouse model for *Twinkle* helicase has been developed (188). Loss of *Twinkle* caused embryonic lethality (approximately E8.5), while tissue specific disruption of *Twinkle* in heart and skeletal muscle tissues caused premature death (approximately 19 weeks) with severe progressive mtDNA depletion and profound respiratory chain dysfunction in heart tissue (188). Earlier studies have shown that mouse models overexpressing mutant forms of *Twinkle*, commonly named as the "Deletor mice" showed adPEO and late onset mitochondrial disease with

mitochondrial myopathy, abnormal skeletal muscle fiber size, COX negative fibers, accumulation of deleted mtDNA and mtDNA depletion (189-191).

MPV17 deficiency: *Mpv17* knockout mice (*Mpv17*$^{-/-}$) were developed and showed profound mtDNA depletion in the liver (154). A mtDNA depletion was also observed in skeletal muscle to a lesser extent, but not in kidney and brain tissues up to 1 year of age. However, 18 months and older mice developed focal segmental glomerulosclerosis with high proteinuria, and severe mtDNA depletion in glomerular tuffs (192). dNTP pools measured in liver, brain and kidney mitochondria of *Mpv17*$^{-/-}$ mice showed marked decrease in the liver dTTP and dCTP pools causing severe mtDNA depletion, while brain and kidney dNTP pools remained unaltered. MPV17 is therefore believed to regulate the mitochondrial nucleotide salvage pathway (193).

4.3 CANCER CELL METABOLISM

Cells convert the biochemical energy from nutrients to ATP, a process known as cellular respiration. Cellular ATP is produced via two interconnected pathways; glycolysis (an anaerobic pathway) and OxPhos (an aerobic pathway). Under aerobic conditions, glucose is broken down to pyruvate in the cytosol, which is then converted into acetyl coenzyme A (acetyl CoA) in the mitochondrial matrix, catalyzed by pyruvate dehydrogenase. The acetyl CoA enters the tricarboxylic acid cycle (TCA cycle or Krebs cycle or citric acid cycle) within the mitochondrial matrix where it is oxidized to carbon dioxide. The TCA cycle generates electron carriers such as NADH and $FADH_2$ that transfer electrons to the mitochondrial respiratory chain (MRC) complexes to generate ATP via OxPhos. Under anaerobic conditions, the pyruvate is fermented to form lactate.

Cancer cells are cells that undergo uncontrolled cell division due to activation or suppression of genes involved in regulating cell growth. One of the features of cancer cell metabolism is the increased preference for glycolysis over OxPhos, even in the presence of oxygen, a process known as aerobic glycolysis or the Warburg effect (194). In order to compensate for the metabolic reprogramming, cancer cells take up higher amounts of glucose and increase lactate production. Glycolysis contributes to more than just ATP synthesis in a cell. Intermediates from the glycolysis and the TCA cycle are essential for several anabolic pathways such as the pentose phosphate pathway and the synthesis of fatty acids, cholesterol, glycogen, glycerol and amino acids (figure 3).

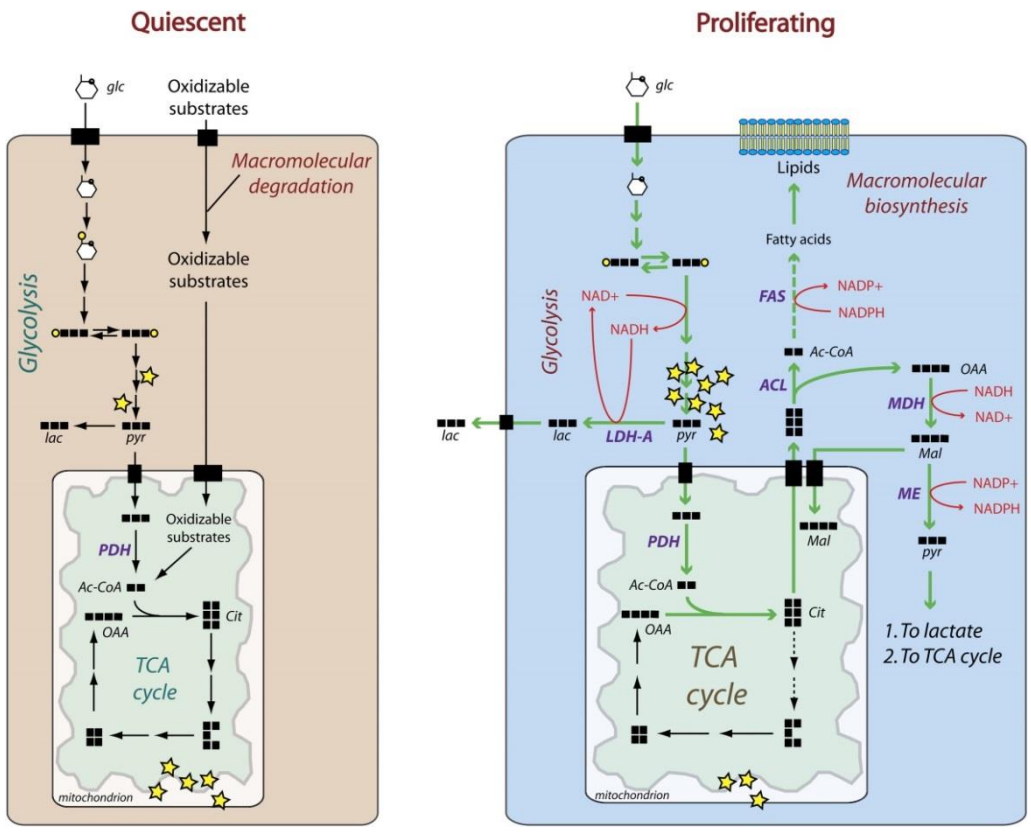

Figure 3: Glucose metabolism in quiescent and dividing cells. Reprinted with permission from Elsevier (195).

Cancer is driven by several factors such as activation of oncogenes, loss of tumor suppressors and mutations in the nDNA or the mtDNA affecting the MRC complexes. Beyond energy production, mitochondria influence cancer metabolism and tumorigenesis in many different ways including to maintain redox homeostasis, to regulate apoptosis, to regulate cellular metabolites and the signaling processes (196).

Alterations in cellular metabolism occur during tumorigenesis to facilitate cell survival and growth. Studies on metabolic profiling of cancer cells have shown that numerous metabolic enzymes and metabolites have tumor specific changes in expression profiles (197-199). Activities of these enzymes and metabolites are a tightly regulated network within the cell and any disturbance may cause cascading effects on the network contributing to the malignant phenotype. Therefore, to target metabolic pathways is important to understand the regulation of metabolic reprogramming in cancer.

4.3.1 Mitochondrial solute carriers

Healthy cells depend on the finely tuned channeling of metabolic substrates and products across subcellular compartments by a number of transporters (200). The mitochondria host several transporters that facilitate transport of substrates across the mitochondrial membrane. The outer mitochondrial membrane contains voltage dependent anion channels, and is relatively permeable, while the inner mitochondrial membrane is highly impermeable in order to maintain efficient OxPhos. The mitochondrial carriers are a family of nuclear encoded proteins called the solute carrier family 25 (SLC25). The SLC25

proteins are localized in the impermeable mitochondrial inner membrane and are essential for effective mitochondrial–cytosolic crosstalk. The SLC25 transporters consists of 53 members that are involved in transport of molecules in several metabolic pathways such as the TCA cycle, the urea cycle, the OxPhos, the gluconeogenesis, the fatty acid oxidation, the amino acid degradation, the maintenance of dNTP pools, the calcium signaling and the iron metabolism (200,201). Based on their substrate the transporters can be broadly classified into different clades as amino acid carriers, nucleotide carriers, uncoupling protein carriers and carboxylate carriers (201).

The mitochondrial SLC25 member 10 (SLC25A10) is a dicarboxylate carrier that transports TCA cycle intermediates between the cytoplasm and the mitochondria (202). The carrier transports dicarboxylates such as malate or succinate across the mitochondrial membrane in exchange for phosphates, succinate and thiosulphates (203-206). In humans, SLC25A10 is highly expressed in liver and kidney tissues, where it plays a major role in gluconeogenesis, the urea cycle and sulphur metabolism (207). In mice, predominant expression of the dicarboxylate carrier is observed in white adipose tissue with a role in fatty acid biosynthesis (208). Fatty acid synthesis occurs in the cytosol and is initiated by the export of citrate from the mitochondria to the cytosol by a mitochondrial citrate carrier SLC25A1 (209) (figure 4).

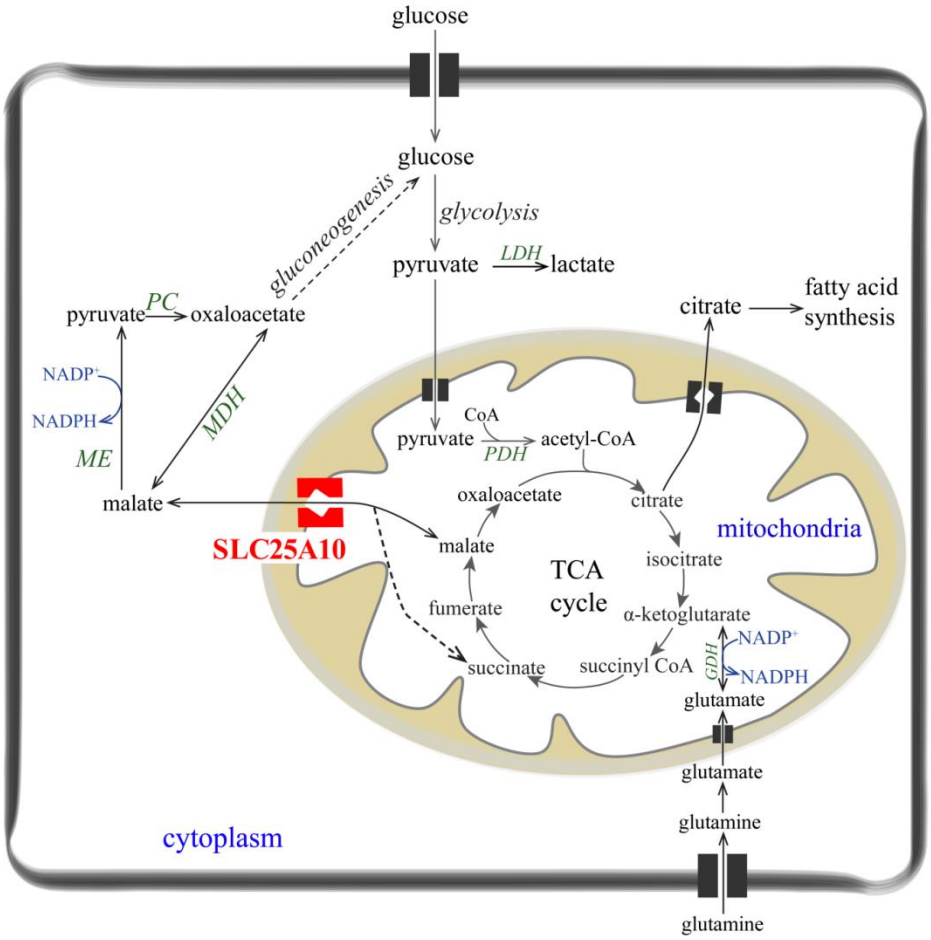

Figure 4: Schematic representation of the SLC25A10 carrier in cell metabolism. Enzymes are represented in green; GDH-glutamate dehydrogenase, LDH-lactate dehydrogenase, MDH-malate dehydrogenase, ME1-malic enzyme 1, PC-pyruvate carboxylase.

SLC25A10 has been shown to transport malate into the mitochondria during the citrate transport to the cytoplasm required for fatty acid synthesis. Inhibition of the SLC25A10 carrier was shown to reduce lipid accumulation in adipose tissues (202). Additionally, SLC25A10 plays a role in the regulation of glucose-stimulated insulin secretion in pancreatic beta cells (210) Overexpression of the SLC25A10 carrier in a human embryonic kidney cell line resulted in hyperpolarization of the mitochondria (208), and overexpression in adipocytes resulted in increased ROS production (211). The dicarboxylate carrier along with the mitochondrial 2-oxoglutarate carrier facilitates glutathione transport from the cytosol to the mitochondria (212,213). Together, these studies show that the SLC25A10 carrier plays an important role not only in providing substrates for several biosynthetic pathways but also in regulating redox homeostasis.

5 TREATMENT STRATEGIES FOR MITOCHONDRIAL DYSFUNCTION

Mitochondrial dysfunction and subsequent OxPhos defects are characteristic of many neurological diseases such as Alzheimer's and Parkinson's disease, diabetes, ageing, cancer and the different forms of mitochondrial diseases that arise due to dysfunctional respiratory chain complexes (214-216). Multiple tissues are affected by defects in the mitochondria, especially tissues that have a high energy demand such as skeletal muscle and brain. Reduction of the metabolic load by dietary manipulation, enzyme replacement, removal of toxic metabolites and organ transplantation are some of the therapeutic approaches for mitochondrial diseases (reviewed in (217)). The mtDNA are constantly subjected to damage due to ROS production within the mitochondria. Studies using dichloroacetate, creatine, coenzyme Q_{10}, antioxidants and lipoic acid have been investigated in patients with mitochondrial diseases (217). The heterogeneity of mitochondrial disorders makes it a challenging task for development of therapeutic approaches.

Currently there are no proven effective treatments for MDS. Care and management of MDS include supportive treatments with vitamins and cofactors, but with poor efficacy. Liver transplantation has shown to improve quality of life in some patients with *POLG1*, *DGUOK* and *MPV17* mutations. However, liver transplantation alone is in most cases not sufficient since the disease manifestations are multisystemic and also involve severe neurological symptoms (159,218-221). A controlled diet avoiding hypoglycemia or a lipid rich diet together with succinate and coenzyme Q_{10} have shown to delay progression of liver disease in some patients with *MPV17* mutations (222,223). Enzyme replacement therapy using allogenic stem cell transplantation and continuous ambulatory peritoneal dialysis have shown promising effects in MNGIE patients (224-227). *In vitro* studies have demonstrated that supplementation of medium with dAMP and dGMP in patient derived dGK deficient quiescent fibroblasts could partially restore the mtDNA depletion (228,229). Recently, studies have shown that oral supplementation of deoxypyrimidine monophosphates (dTMP and dCMP) was able to prolong the lifespan and delay disease onset in a $Tk2^{-/-}$ knockin

mouse model (230). The effect correlated with the dose of administration and an increase in mtDNA copy number and respiratory chain activities were observed in brain, heart, skeletal muscle, kidney and liver in these mice (230). Gene therapy approaches using adeno associated virus and lentiviral vectors have been studied in mouse models for ethyl malonic encephalopathy and the MNGIE form of MDS (231,232).

Current therapies for cancer include radiation and chemotherapy that have adverse effects on all cells. Targeted therapy is a newer type of cancer therapy that specifically targets cancer cells with potentially less side effects on normal healthy cells. There is a need for additional treatment strategies to make rational combination approaches possible. With the development of inhibitors of metabolic enzymes it could be possible to target the metabolic reprogramming in cancer cells. Characteristics of cancer cells are upregulated glycolysis and increased lactate production. Many compounds targeting key metabolic enzymes, intermediates and transporters of the glycolytic pathway are exploited for development of therapeutic strategies (233-235). Glutamine is a multifunctional metabolite that is involved in energy production, synthesis of macromolecules and regulation of redox homeostasis. Several cancer types such as the ones driven by Myc and Kras mutations are highly sensitive to glutamine deprivation (236,237). Approaches that target the different roles of glutamine metabolism and dependency have been studied (reviewed in (238)). Antioxidants are important to regulate ROS mediated mitochondrial damage and are explored as possible anticancer agents. Cancer cells increase their antioxidant capacity to prevent buildup of ROS. The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) generated in the cytosol primarily via the pentose phosphate pathway, is involved in maintaining the antioxidant defense systems by quenching the ROS produced during rapid cell proliferation. NADPH donates electrons for the ROS scavenging systems including glutathione and thioredoxin (239). *In vitro* studies have demonstrated that targeting enzymes that regulate redox balance are effective against certain cancers cell lines (240-242).

The importance of mitochondria and mtDNA depletion for tumorigenesis and metastasis has several implications in terms of future cancer treatment including identification of selective drug targets and development of new intervention strategies. To understand the regulation of cancer metabolism could serve as a platform to design and predict the efficacy of different therapies.

6 AIM OF THE PRESENT WORK

The aim of the present study was

- to get mechanistic insights in the mitochondrial dysfunction caused by TK2 deficiency
- to elucidate possible treatment strategies for TK2 deficiency and other mitochondrial disorders
- to investigate the mitochondrial carrier, SLC25A10 and its role in cancer cell metabolism and in the regulation of redox homeostasis.

7 REFLECTION ON THE METHODOLOGY

In general, most of the methods used in this thesis are well established methods. Cell culture techniques, DNA, RNA and protein extraction protocols, genotyping using polymerase chain reaction (PCR), Western blot for protein expression studies, and quantitative real time PCR (qPCR) (both TaqMan and SYBR green techniques) for gene expression profiling, are all described in detail in papers I, II, III and IV. Microscopic analysis of mouse tissues using histopathology, immunohistochemistry and electron microscopy was performed at the Department of Laboratory Medicine, Division of Pathology, Karolinska Institute. Several kits used in all four papers are listed in table 2.

Table 2: List of kits used in the thesis

Method	Kit	Company
DNA extraction	DNeasy blood and tissue kit	Qiagen
PCR	GoTaq G2 Flexi DNA Polymerase	Promega
	PfuUltra high fidelity DNA polymerase	Agilent
RNA extraction	RNeasy mini kit	Qiagen
cDNA synthesis	high capacity cDNA reverse transcription kit	Applied Biosystems
qPCR	TaqMan universal qPCR mix	Applied Biosystems
	KAPA SYBR Fast qPCR kit	KAPA Biosystems
	ABI 7500 Fast Machine	Applied Biosystems
siRNA vector	pSilencer^{TM} Puro Expression Vectors kit	Applied Biosystems
siRNA transfection	Fugene 6 HD kit	Promega
NADP/NADPH assay	NADP/NADPH assay kit	Abcam
XTT asssay	Cell proliferation assay kit II	Roche Life Science

7.1 TRANSGENE CONSTRUCTS

The *Dm*-dNK transgene construct was generated using the previously cloned *Dm*-dNK cDNA (66). The 850 bp open reading frame sequence was ligated to a pcDNA3 vector with mouse cytomegalovirus (CMV) promoter region (paper I and II) and with mouse albumin promoter (paper III). A 6-histidine tag was fused to the C-terminal of the *Dm*-dNK sequence in both constructs. The transgene constructs were digested from pcDNA3 vector using *Bgl*II and *Dra*III, and *SnaB*I and *Dra*III restriction enzymes for CMV-*Dm-dNK* and albumin-*Dm-dNK* constructs respectively. The two transgenic constructs are shown in figure 5.

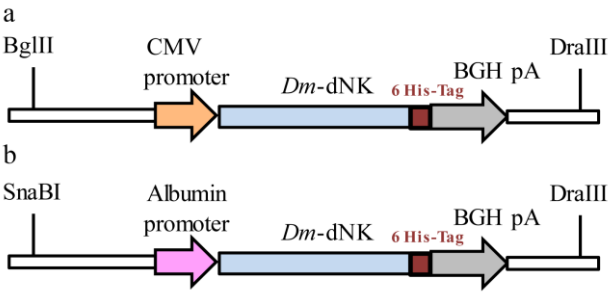

Figure 5: Dm-dNK transgenic constructs. (a) CMV-Dm-dNK transgenic construct, (b) albumin-Dm-dNK transgenic construct

7.2 MICE

Wild type (C57BL6/N) mice from Charles River Laboratories were used in this thesis. Two different transgenic mouse strains have been generated; CMV-*Dm*-dNK and albumin-*Dm*-dNK. The CMV-*Dm-dNK* mice were intercrossed with the previously described *Tk2* knockout mice ($Tk2^{-/-}$) (168) to generate $Dm\text{-}dNK^{+/-}Tk2^{-/-}$ mouse strain. These mice were used to study whether transgenic *Dm*-dNK expression was able to rescue *Tk2* deficiency in mice. The albumin-*Dm*-dNK mice will serve as a tool to study whether tissue specific expression of *Dm*-dNK is able to rescue Tk2 deficiency in mice.

All mice were housed and bred at the Karolinska Institute, Division of Comparative Medicine, Clinical Research Center, Huddinge. All animal procedures were compliant with the Swedish Board of Agriculture (Jordbruksverket) animal research ethical regulations (ethical permits S135-11, S6-13, S100-15). Transgenic mice were generated using pronuclear injection technique performed at the Division of Comparative Medicine, Clinical Research Center, Huddinge for CMV-*Dm-dNK* mice and Karolinska Center for Transgenic Technologies (KCTT), Solna for the albumin-*Dm-dNK* mice.

7.3 MITOCHONDRIAL DNA COPY NUMBER

To study the effect of *Dm*-dNK in *Tk2* deficient mice, mtDNA copy number was measured in several tissues of wild type and *Dm*-dNK expressing mice. Total DNA was extracted from mouse tissues and mtDNA copy number was performed using qPCR. Specific TaqMan primers and probes were designed for a nuclear encoded ribonuclease P RNA component H1 *(Rpph1)*, and mitochondrial encoded NADH dehydrogenase 1 (*mt-ND1)*. mtDNA was quantified using standards that were prepared using the pGEMT plasmid containing one copy each of the mouse *Rpph1* and *mt-DN1* genes. The mtDNA copy number was measured in skeletal muscle, brain and liver of all mice (paper I and II).

7.4 MEASUREMENT OF dNTP POOLS

dNTPs were extracted from whole cell extracts (paper I and II) and mitochondria (paper II) using MTSE buffer and methanol (described in detail in paper I). dNTP measurement was performed by a DNA polymerase based assay using templates and primers designed specifically for the different dNTPs measured. (243). The technique is based on

incorporation of tritium labelled dATP (^{3}H-dATP) (for measuring dTTP, dCTP, dGTP pools) or ^{3}H-dTTP (for measuring dATP pools) into a template oligonucleotide via primer extension. The DNA polymerase used for this reaction is the Klenow subunit of *E.coli* polymerase I, known as the Klenow polymerase. The assay was carried out by incubating extracts containing unknown amounts of all 4 dNTPs with the Klenow polymerase, tritiated dNTP and a template specific for the dNTP to be measured, with a known repetitive deoxyribonucleotide sequence. The reaction was incubated for 30-45 min, spotted on Whatman DE-81 filter discs and washed with Na_2HPO_4, water and ethanol. The retained radioactivity was measured by scintillation counting using beta counters. In paper I, dCTP, dTTP and dGTP pools were determined using this technique. It was not possible to measure dATP pools because, *Dm*-dNK expression causes a large increase in dTTP pool which affects the dATP pool measurements. In paper II, a modified template strand was synthesized for measurement of the dATP pools.

7.5 MUTATION ANALYSIS

The mtDNA are known to have higher acquired mutation rates than nDNA due to constant exposure to ROS, lack of histones and inefficient DNA repair mechanisms (244). The mitochondrial NCR is a hotspot for mutagenic effects in mtDNA as it contains two hypervariable regions. To sequence the mitochondrial NCR is therefore a great tool to study mtDNA mutation frequencies. Expression of *Dm*-dNK in mouse tissues causes increase in all four dNTP pools, particularly dTTP. Analysis of mtDNA point mutations in mitochondrial *NCR* and cytochrome b (*Cytb)* gene was performed to study the effect increased dTTP pools in *Dm*-dNK$^{+/-}$ mice (paper I). Total DNA was extracted from skeletal muscle of wild type and *Dm*-dNK$^{+/-}$ mice using DNeasy kit (Qiagen). Fragments of the *mt-NCR* and *mt-Cytb* were amplified using high-fidelity PCR and cloned into the pGEM-T vector (Promega) according to manufacturer's instructions. Multiple clones obtained were sequenced, and point mutations and mutation frequencies were calculated.

Mutation frequencies were measured in the well characterized hypoxanthine-guanine phosphoribosyl transferase encoded by the *HPRT* gene. The *HPRT* gene locus has been used as a tool for mutagenesis studies for many years owing to the fact that a wide range of mutations are associated with it. In order to detect point mutations in the *Hprt* exon sequence, the mouse *Hrpt* mRNA and corresponding cDNA was used as starting point of the sequencing analysis (paper II). mRNA was extracted from skeletal muscle of 12 month old wild type and *Dm*-dNK expressing mice (both *Dm-dNK*$^{+/-}$ and *Dm-dNK*$^{+/-}$*Tk2*$^{-/-}$), and cDNA synthesis was performed using high capacity cDNA reverse transcription kit (Applied Biosystems). Fragments of mouse *mt-Cytb* gene and *Hprt1* gene were amplified using high-fidelity PCR, from cDNA template. The PCR amplicon fragments were cloned to pGEM-T vector (Promega) according to manufacturer's instructions. Multiple clones obtained were sequenced, and point mutations and mutation frequencies were calculated.

8 BRIEF SUMMARY OF RESULTS

Paper I, II and III (manuscript) focus on therapeutic approach for mitochondrial DNA depletion syndrome caused by TK2 deficiency. Paper IV focuses on the role of mitochondrial carriers in cancer cell metabolism and for the regulation of the redox balance in cells. A brief summary of the results is presented below.

8.1 PAPER I

Transgene expression of *Drosophila melanogaster* nucleoside kinase reverses mitochondrial thymidine kinase 2 deficiency

The life span of *Tk2* knockout mice (*Tk2*$^{-/-}$) is approximately 3-4 weeks. These mice die due to severe mtDNA depletion in multiple organs. In an attempt to rescue the severe mtDNA depletion caused by TK2 deficiency, transgenic mice expressing the deoxynucleoside kinase from *Drosophila melanogaster* (*Dm-dNK*$^{+/-}$) driven by the CMV promoter were generated. These mice were crossed with *Tk2*$^{+/-}$ mice to get *Dm-dNK*$^{+/-}$*Tk2*$^{+/-}$ mice, which were then intercrossed with *Dm-dNK*$^{+/-}$*Tk2*$^{+/-}$ mice to obtain *Dm-dNK*$^{+/-}$*Tk2*$^{-/-}$ mice. The *Dm-dNK* expressing mice (*Dm-dNK*$^{+/-}$ and *Dm-dNK*$^{+/-}$*Tk2*$^{-/-}$) were characterized for a period of 6 months. *Dm*-dNK activity was the highest in skeletal muscle and kidney and lowest in liver and heart tissues and was found to be constantly expressed up to the age of 6 months. No difference in mtDNA copy number was observed in skeletal muscle of wild type and *Dm-dNK* expressing mice. *Dm*-dNK expression resulted in very high dTTP levels (>100 fold) and slightly high dCTP and dGTP levels (approximately 3 and 1.5 fold respectively) in the skeletal muscle extracts of *Dm-dNK*$^{+/-}$*Tk2*$^{-/-}$ mice in comparison to wild type mice. There were no major histopathological difference observed in skeletal muscle and liver. Mutation analysis of *mt-Cytb* and *mt-NCR* revealed no significant differences in *Dm-dNK* expressing mice compared to wild type mice.

8.2 PAPER II

Long term expression of *Drosophila melanogaster* nucleoside kinase in thymidine kinase 2 deficient mice with no lethal effects caused by nucleotide pool imbalances

In order to study the long term effects of *Dm-dNK* transgene in mice, the *Dm-dNK*$^{+/-}$*Tk2*$^{-/-}$ mice were studied for their growth and behavior for a period of 20 months. During this period the *Dm-dNK* transgene was constantly expressed. There was a significant decrease in total body weight of the *Dm-dNK*$^{+/-}$*Tk2*$^{-/-}$ mice compared to wild type mice due to decrease in subcutaneous and visceral fat, likely due to the low enzyme activities in some tissues such as liver, heart and adipose tissues. Expression of *Dm*-dNK resulted in increase in all four dNTP levels with dTTP being the highest. This increase in dTTP pools did not cause any significant point mutations in the nuclear or mitochondrial DNA. There was a slight decrease in mtDNA copy number in the *Dm-dNK*$^{+/-}$*Tk2*$^{-/-}$ mice compared to wild type or *Dm-dNK*$^{+/-}$ mice at 12 months of age, however this difference was not observed at 18 months. Electron microscopy of the kidney and muscle did not show any changes in the mitochondrial density or structure.

A slight increase in mRNA levels of thymidine phosphorylase enzyme was observed in the *Dm-dNK* expressing mouse tissues, while all other dNTP metabolizing enzymes were similar to wild type mRNA levels. The decrease in body fat was observed only in *Dm-dNK*$^{+/-}$*Tk2*$^{-/-}$ mice and not in control *Dm-dNK*$^{+/-}$ mice, therefore it is likely to be an effect of lack of *Tk2* and lower expression of *Dm-dNK* in the adipose tissues.

8.3 PAPER III (MANUSCRIPT)

Construction of a mouse strain with liver specific expression of *Drosophila melanogaster* nucleoside kinase

The *Dm-dNK* transgene driven by the CMV promoter could rescue *Tk2* knockout mice, and restore mtDNA depletion caused by *Tk2* deficiency. This study aims to investigate whether *Dm-dNK* expressed solely in the liver would be sufficient to rescue mtDNA depletion caused by deoxynucleoside kinase deficiency. A mouse model was constructed to express *Dm-dNK* specifically in liver tissue driven by the liver specific albumin promoter. 8 out of 50 founder mice (16%) genotyped had the *Dm-dNK* transgene integrated along with the albumin promoter. Only 2 out of 8 positive founder mice had a higher *Dm-dNK* expression (approximately 2.5 times) compared to wild type control. *Dm-dNK* expression was measured both in mRNA level using quantitative real time PCR and in protein level using enzymatic assays. The two founder mice were selected for further studies based on high expression of *Dm-dNK* in liver and low expression in other tissues.

8.4 PAPER IV

The mitochondrial carrier SLC25A10 regulates cancer cell growth

SLC25A10, the mitochondrial dicarboxylate carrier, was knocked down in A549 cells using the siRNA technique. Our results show that knockdown of SLC25A10 in A549 cells changed the growth properties to a less malignant phenotype, with small cell size, monolayer growth and polarized mitochondrial formation around the nucleus. SLC25A10 knockdown cells had a higher dependency on glutamine, and an increased sensitivity to oxidative stress. In dividing cells, knockdown of SLC25A10 caused decreased NADPH/total NADP ratio compared to control cells, in cells grown in both glutamine and glutamine free medium. Gene expression of several genes involved in maintenance of redox homeostasis, metabolic and regulatory enzymes and some genes involved in cancer signaling pathways were analyzed using qPCR. Gene expression of *TXN2* and *TXNRD2* were downregulated while gene expression of *GLUD2*, *LDHA*, and *PDHA1* were upregulated in dividing siRNA knockdown cells compared to control cells. In confluent cells, gene expression of *TXN2*, *LDHA* and *LDHB* were downregulated and gene expression of *TXN*, *TXNRD1*, *GLUD1* and *GLUD2* were up regulated, in siRNA knockdown cells compared to control cells. A decrease in protein expression, measured using Western blot, of some proteins involved in cancer signaling pathways such as p53, HIF1α, and p21 was observed in siRNA knockdown cells compared to control cells.

9 CONCLUDING REMARKS

A *Tk2* deficient mouse model expressing *Dm-dNK* transgene was established and characterized. Transgene expression of *Dm*-dNK reversed mtDNA depletion and rescues the severe phenotype caused by *Tk2* deficiency in the mice. The deoxyribonucleotides synthesized in the cytosol are transported to mitochondria in quiescent cells. The *Dm*-dNK transgenic mouse serves as a model for deoxyribonucleoside gene or enzyme substitutions and dNTP alterations in different tissues.

The expression of *Dm-dNK* in $Tk2^{-/-}$ mice prolonged its life span of from 3 weeks to at least 20 months. The nuclear expression of *Dm-dNK* expanded dNTP pools in the cytosol and mitochondria required for mtDNA synthesis. Normal mtDNA levels were observed in skeletal muscle and liver tissues of the $Dm\text{-}dNK^{+/-}Tk2^{-/-}$ mice. A large increase in the dTTP pools did not cause lethal side effects in these mice.

A mouse model with liver specific expression of the *Dm-dNK* transgene was established. Two founder mice have been characterized and will further be crossed with the *Tk2* knockout mice. This mouse model will address the questions on whether a tissue specific expression would be able to rescue the mtDNA depletion caused by *Tk2* deficiency.

The mitochondrial carrier SLC25A10 knockdown cells changed its growth properties to a less malignant phenotype. The SLC25A10 knockdown cells were more vulnerable to glutamine deprivation and lead to oxidative stress. Gene expression of genes involved in metabolic regulatory pathways and redox balance were altered in SLC25A10 knockdown cells. The metabolic alterations were linked to an energy metabolic shift from glycolysis to mitochondrial OxPhos. The SLC25A10 carrier plays an important role in regulating cancer cell redox homeostasis.

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